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Changes in glucose metabolism during diabetes are linked to an increased risk for the development of breast cancer. Aldose reductase, the rate-limiting polyol pathway enzyme that converts glucose into sorbitol, not only is an important mediator of the pathologies associated with diabetes, but is also upregulated in many cancer cells and may be involved in cancer cell resistance to chemotherapeutic drugs. Furthermore, increased expression of aldose reductase in cancer cells may reduce the effectiveness of chemotherapeutic compounds by increasing drug metabolism or by decreasing drug uptake. Thus, these studies addressed the hypothesis that inhibition of aldose reductase enhances cell sensitivity to anti-cancer drugs. Using the specific aldose reductase inhibitor, ethyl 1-benzyl-3-hydroxy-2(5H)-oxopyrrole-4-carboxylate (EBPC), experiments tested whether aldose reductase inhibition could enhance the cytotoxic effects of the anti-cancer agents doxorubicin or cisplatin in Hela cervical carcinoma and MDA-MB-468 breast cancer cells. Cell growth and death assays revealed that co-administration of aldose reductase inhibitors increased the cytotoxic effects of chemotherapeutic drugs on cervical and breast cancer cells. In summary, these data provide evidence to support further studies testing the use of aldose reductase inhibitors as adjuvant therapy to improve the effectiveness of existing chemotherapeutic drugs.

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Table of contents

Cover..... 1

SF 298..... 2

Introduction..... 4

Body..... 4

Key research accomplishments..... 5

Reportable outcomes..... 5

Conclusions..... 5

References..... 6

Appendices..... 6

Introduction

Changes in glucose metabolism during diabetes are linked to an increased risk for the development of cancer (Talamini et al., 1997; Weiderpass et al., 1997). Aldose reductase is a metabolic enzyme that converts glucose into sorbitol and has been implicated in causing neuropathies associated with diabetes. Moreover, aldose reductase is often found to be upregulated in cancer cells, including more aggressive forms of breast cancer (Hyndman and Flynn, 1999). Thus, the overall goal of this proposal was to determine whether inhibition of the aldose reductase could be a feasible approach to enhance the effectiveness of existing chemotherapeutic drugs used to treat breast cancer. Cultured cancer cell lines were used to test the effects of aldose reductase inhibitors on doxorubicin or cisplatin-mediated cytotoxicity. Using a variety of methods for assessing cell growth and cell death, our findings suggest that aldose reductase inhibitors increase the effectiveness of chemotherapeutic drugs. Furthermore, these data support further studies in animal models examining the use of aldose reductase inhibitors as adjuvant therapy for chemotherapy.

Body

The goal of this proposal was to determine whether inhibitors of aldose reductase increase chemotherapeutic drug effectiveness and to identify intracellular signaling pathways that might be regulated by increased aldose reductase expression in breast cancer cells. Initial studies used a Hela cervical carcinoma cell line because these cells express high levels of aldose reductase. These studies demonstrated that concomitant inhibition of aldose reductase increased the cell death response to doxorubicin and cisplatin. The details of these experiments are outlined in the manuscript "Inhibition of aldose reductase enhances Hela cell sensitivity to chemotherapeutic drugs and involves activation of ERK MAP kinases", which was recently accepted by the journal ***Anti-Cancer Drugs*** and is provided as a preprint in the appendix.

The proposed studies hypothesized that increased aldose reductase expression in cancer cells may serve to protect growth regulating signaling pathways and thus promote a growth advantage in cancer cells. Our laboratory is interested in the function of the extracellular signal-regulated kinase (ERK) signaling pathway, which plays an integral role in regulating cell proliferation. Interestingly, inhibition of aldose reductase caused an activation of the ERK signaling pathway and this activation was required for the increased cytotoxic response. These findings are also more thoroughly discussed in the manuscript in the appendix.

Since the ultimate goal of this proposal is to determine the feasibility of aldose reductase inhibitors in enhancing cytotoxicity of anti-cancer drugs in breast cancer, subsequent studies were performed using breast cancer cell lines that varied in metastatic potential. The expression of aldose reductase protein in the aggressive MDA-MB-468 cell line was significantly higher than the less aggressive MCF7 breast cancer cell line. In support of our hypothesis, treatment of MDA-MB-468 cells with aldose reductase inhibitors significantly enhanced the cytotoxic effects of doxorubicin or cisplatin. Unlike the Hela cells, activation of the ERK signaling pathway did not appear to be involved in the enhanced cytotoxic effects induced by the aldose reductase inhibitors. Current studies are examining growth and survival pathways that are regulated by phosphatidylinositol-3 kinase (PI3K). Preliminary data indicates that inhibitors of aldose reductase also inhibit PI3K-dependent signaling pathways, which is consistent with our hypothesis that aldose reductase protects signaling events that promote cell growth and survival.

Key research accomplishments

* Demonstrated the effectiveness of aldose reductase inhibitors in enhancing the cytotoxic effects of commonly used chemotherapeutic drugs, such as doxorubicin and cisplatin.

* Demonstrated that in Hela cervical carcinoma cells aldose reductase inhibitors increase the toxic effects of doxorubicin and cisplatin through a mechanism that involves activation of the ERK MAP kinase pathway.

Reportable outcomes

The manuscript titled "Inhibition of aldose reductase enhances Hela cell sensitivity to chemotherapeutic drugs and involves activation of ERK MAP kinases" was recently accepted by the journal ***Anti-Cancer Drugs*** on 6/18/02.

Conclusions

Our findings indicate that aldose reductase inhibitors enhance the effectiveness of existing chemotherapeutic drugs and support further testing of aldose reductase inhibitors for use as adjuvant drugs for treating breast cancer. In addition, we believe these findings could support the use of aldose reductase inhibitors to decrease the amount of doxorubicin or cisplatin that is now being used clinically for treating cancer, yet still be effective in eliminating cancer cells. This could potentially benefit patients by reducing many of the debilitating toxic side effects associated with these anti-cancer drugs. We have recently submitted an Idea award grant to the Department of Defense, which will enable us to continue these studies if funded. Proposed studies are aimed at determining the effects of aldose reductase inhibition on chemotherapeutic drug uptake and metabolism. In addition, we will continue to examine how aldose reductase activity and sorbitol production may influence signaling pathways that promote cell growth and survival. For example, proposed experiments will determine whether aldose reductase inhibitors affect ERK and PI3K-dependent signaling pathways. These studies will focus only on breast cancer cells incorporating both cell and animal models. Importantly, aldose reductase inhibitors have already been tested in clinical trials for treatment of neuropathies associated with diabetes. Thus, many of the pharmacokinetic and toxicological parameters for these drugs have been examined. If our proposed studies prove our hypothesis to be correct, the use of aldose reductase inhibitors could then be rapidly be transferred to clinical trials for the treatment of cancer.

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Appendices:

A preprint of the manuscript entitled "Inhibition of aldose reductase enhances Hela cell sensitivity to chemotherapeutic drugs and involves activation of ERK MAP kinases" is enclosed. This paper was accepted by the journal ***Anti-Cancer Drugs*** on 6/18/02.

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Inhibition of aldose reductase enhances Hela cell sensitivity to chemotherapeutic drugs and involves activation of ERK MAP kinases.

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Keywords: MAP kinase, cytotoxicity, aldose reductase, chemotherapy, polyols

Abbreviations used: EBPC; ethyl 1-benzyl-3-hydroxy-2(5H)-oxopyrrole-4-carboxylate, MAP kinase; mitogen activated protein kinase, ERK; extracellular signal-regulated kinase, MTT; 3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium bromide, PARP; poly ADP-ribose polymerase.

Summary

Changes in glucose metabolism during diabetes are linked to an increased risk for the development of cancer. Increased activity of aldose reductase, the rate-limiting polyol pathway enzyme that converts glucose into sorbitol, mediates pathologies associated with diabetes and is thought to be involved in increased resistance to chemotherapeutic drugs. Thus, increased intracellular sorbitol levels may serve a protective function in cancer cells. In these studies we determined whether an inhibitor of aldose reductase could enhance the effectiveness of anti-cancer agents. Our findings indicate that treatment with the aldose reductase inhibitor, ethyl 1-benzyl-3-hydroxy-2(5H)-oxopyrrole-4-carboxylate (EBPC) enhances the cytotoxic effects of the anti-cancer agents doxorubicin and cisplatin in Hela cervical carcinoma cells. To establish a mechanistic basis for the increased cytotoxicity by EBPC, we examined the activity of the extracellular signal-regulated kinase (ERK) pathway, which is an important regulator of cell growth. Interestingly, treatment with EBPC in combination with the chemotherapeutic drugs increased ERK activity as compared to treatment with the chemotherapeutic drugs, suggesting a possible role for the ERK pathway in mediating doxorubicin or cisplatin-induced cell death. Consistent with this possibility, inhibition of ERK activation by the MEK inhibitor, U0126, reversed the EBPC-mediated enhancement of cell death. In summary, these data provide evidence that adjuvant therapy with aldose reductase inhibitors improve the effectiveness of chemotherapeutic drugs, possibly through an ERK pathway mediated mechanism.

Introduction

Resistance to chemotherapeutic drugs and the debilitating side effects associated with increasing doses of chemotherapy remain a major barrier for the effective treatment of cancer. Several studies indicate that changes in glucose metabolism correlate with increased tumorgenicity, chemotherapeutic drug resistance, and cytotoxic immunologic responses [1,2]. In addition, the correlation between diabetes mellitus and the development of endometrial and breast cancer suggests that changes in glucose metabolism contribute to enhanced cell proliferation [3,4]. Furthermore, chemotherapeutic toxicity and counter-therapeutic effects may be mediated by de-regulation of glucose-responsive proteins or through upregulation of proteins that promote glucose transport and metabolism [5,6]. Therefore, proteins that are involved in glucose metabolism may be targeted for developing effective chemotherapeutic protocols.

Increased flux of glucose through the polyol pathway (also called the sorbitol pathway) has been linked to a number of diabetes complications including neuropathy and retinopathy [7]. Aldose reductase is the initial rate limiting polyol pathway enzyme that converts glucose to sorbitol [8,9]. Thus, aldose reductase inhibitors are currently being tested in human trials and may provide some level of benefit for treating diabetic neuropathy [10,11,12]. In addition, aldose reductase activity has been linked to cell growth. For example, aldose reductase is reportedly involved in glucose-induced proliferation of smooth muscle cells [13]. Furthermore, increases in aldose reductase protein have also been observed in carcinoma cells [14,15]. Importantly, it has been suggested that increased aldose reductase expression and intracellular sorbitol accumulation may be partly responsible for reduced efficacy of chemotherapeutic drugs used to treat breast cancer [6]. In such cases, aldose reductase activity appears to influence cell growth and the response of tumor cells to anti-cancer drugs. The mechanistic basis for aldose reductase activity,

sorbitol production, and tumor cell growth is unclear, but may involve activation of growth and survival signals, osmotic sensing signaling pathways, and the regulation of gene expression.

The three major families of mitogen activated protein (MAP) kinase pathways that regulate cell growth and stress responses include the extracellular signal-regulated kinases (ERKs), c-Jun amino terminal kinases (JNKs) and p38 proteins [16]. The ERK MAP kinases function primarily in regulating cell proliferation and differentiation, whereas, the JNK and p38 MAP kinases mediate stress and apoptotic signals [16]. However, in some cases ERK pathway activation has been linked to the induction of cell death [17]. Thus, depending on the extracellular signals and cell type, MAP kinase pathways may function in promoting or inhibiting cell growth. Increased extracellular glucose concentrations activate the ERK and p38 MAP kinase pathways in several cell and animal models of diabetes [18, 19]. In addition, animal models of long-term diabetes show higher basal levels of ERK activity compared to non-diabetic controls [20]. Whether MAP kinase activation functions in promoting or protecting against the effects of high glucose concentrations during diabetes is not yet known. Similarly, ERK activation in response to changes in glucose metabolism could function in promoting cell survival and proliferation of cells susceptible to transformation.

In these studies, we addressed whether aldose reductase activity functions in sensitizing cells to the genotoxic chemotherapeutic agents, doxorubicin and cis-platinum(II)diammine dichloride (cisplatin). We find that inhibition of aldose reductase in combination with doxorubicin or cisplatin treatment enhances the cell death response of cultured cancer cells. Our data also indicate that inhibition of aldose reductase enhances ERK activity, and that this activity is required for the enhanced cell death observed in the presence of the aldose reductase inhibitor. Thus, in contrast to its role in promoting cell growth and in agreement with previous studies [17],

ERK activity also can mediate cell death. These data provide a rationale for further study of the use of aldose reductase inhibitors as adjuvant therapy for the treatment of cancer.

Materials and Methods

Cell culture: Cervical adenocarcinoma (Hela) cells were grown in complete media (Dulbecco's Modified Eagles Medium (DMEM)+10% fetal bovine serum (FBS), supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). For protein analysis following treatments, cells were trypsinized and seeded onto 6-well (35mm) plates and allowed to grow for 24 hours. Cells were then cultured in the absence or presence of doxorubicin at doses ranging from 0.1 to 10 μg/ml or treated with 0-240μM of cisplatin. In some experiments, cells were also treated in the absence or presence of aldose reductase inhibitors at concentrations of 10-60μM. Treated and untreated cells were further incubated for 2-48 hours prior to harvesting and analysis.

Reagents. The aldose reductase inhibitor, ethyl 1-benzyl-3-hydroxy-2(5H)-oxopyrrole-4-carboxylate (EBPC), was purchased from Tocris Cookson Inc. (Ballwin, MO) and stored as a 10 mM stock solutions in ethanol. Stock solutions of doxorubicin-hydrochloride (10mg/ml), cis-platinum(II)diammine dichloride (cisplatin, 60mM), and MTT (3-(4,5-dimethyl-2-thiazoyl)-2,5-diphenyltetrazolium bromide, 5mg/ml) were purchased from Sigma and stored in DMSO (doxorubicin and cisplatin) or phosphate buffered saline (MTT). The MEK1/2 inhibitor, U0126, was purchased from Promega and stored at a 10mM stock solution in DMSO. The monoclonal antibody recognizing phosphorylated ERK1/2 (pT183, pY185) was purchased from Sigma. Polyclonal antibodies recognizing poly (ADP-ribose) polymerase (PARP, H-250) and ERK2 (C-14) were purchased from Santa Cruz Biotech.

Cell proliferation assays. Cell proliferation assays were performed using direct cell counts following trypan blue staining or by MTT assay. For cell counts using trypan blue, cells were seeded on 12 well plates and incubating in the absence or presence of doxorubicin, cisplatin, or aldose reductase inhibitors for 24-48 hours. Floating and adherent cells were collected following

trypsinization, stained with trypan blue (0.2%) in phosphate buffered saline, and counted using a hemocytometer. Dead cells were scored by their failure to exclude trypan blue and expressed as a percentage of the total number of cells counted under each condition. For the MTT assay, cells were seeded on 24 well plates containing 1ml of media and treated as indicated above for the trypan blue assay. Following treatments, 100μl of MTT stock was added to each well and incubated for 3 hours. The reaction mixture was stopped with DMSO, transferred to a 96 well plate and absorbance was measured at 570nm to quantify the formazan product and 690nm for background levels using a multi-well plate reader (Multiskan Ascent, MTX Lab Systems, Vienna Va).

Immunoblotting. Untreated and treated cells were washed twice with cold phosphate buffered saline, lysed with 300 μl of tissue lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 2 mM sodium pyrophosphate, 10% glycerol, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine), and centrifuged at 15,000 RPM to clarify lysates. Lysates (~20 μg of total protein) were diluted with an equal volume of 2X SDS-sample buffer and resolved by SDS-PAGE. Proteins were transferred to PVDF membrane, blocked for 1-2 hr with 5% nonfat dry milk in Tris-buffered saline (TBS, 50 mM Tris, pH 7.5, 0.15 M NaCl, and 0.1% Tween-20), and incubated with primary antibodies diluted in TBS+1% BSA for 2 hr to overnight. Membranes were washed several times in TBS and incubated with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Jackson ImmunoResearch, diluted 1:10,000). Protein immunoreactivity was detected by enhanced chemiluminescence (NEN/DuPont).

Statistics. Statistical significance between treatment groups was determined using analysis of variance.

Results

Induction of cell death in response to varying doses of doxorubicin and cisplatin. Initial experiments characterized the cytotoxic effects of doxorubicin or cisplatin on Hela cells. Cells were plated and allowed to grow for 24 hours before being exposed to varying concentrations of doxorubicin or cisplatin for an additional 24-48 hours. Dead cells that failed to exclude trypan blue dye were counted and expressed as a percentage of the total cells and cell proliferation was determined using MTT assays under each condition. Cells showed a dose- and time-dependent cytotoxic response to both doxorubicin and cisplatin (figure 1). After a 2 day exposure to drugs, cells treated with cisplatin showed increased cell death with increasing drug doses, whereas, the maximum cell death as measured by trypan blue dye exclusion occurred with approximately 0.25μg/ml for doxorubicin (figure 1A and B). Cell proliferation was also inhibited in a dose-dependent manner following a 2 day exposure to doxorubicin or cisplatin as measured using the MTT assay (figure 1C and D). Similar to the dead cell counts in figure 1A and B, a 1 day exposure to drugs showed only a smaller degree of cell growth inhibition at the highest concentrations as measured by the MTT assay (figure 1C and D).

Induction of poly ADP-ribose polymerase (PARP) cleavage with varying doses of cisplatin and doxorubicin. The cleavage of poly (ADP-ribose) polymerase (PARP), as an indicator of apoptotic cell death, was examined in cells treated for up to 28 hours with varying doses of doxorubicin or cisplatin. Doxorubicin or cisplatin caused a dose- and time-dependent cleavage of the 112kD full-length PARP protein and the subsequent generation of the 85kD fragment (figure 2). The concentrations of doxorubicin or cisplatin that stimulated PARP cleavage correlated well with the drug concentrations that stimulated cell death and inhibited cell proliferation as shown in figure 1. Thus, the experiments in figures 1 and 2 established the

conditions for examining the effects of aldose reductase inhibitors on cisplatin or doxorubicin-mediated cytotoxicity.

Inhibition of aldose reductase enhances cisplatin and doxorubicin-mediated cell death. Since Hela cells express high levels of aldose reductase [21], we chose these cells to test whether the presence of an aldose reductase inhibitor could enhance the cytotoxic effects of anticancer agents. For these experiments, cells were exposed to sub-optimum doses of doxorubicin or cisplatin that typically cause minimal cell death (figure 1) in the presence or absence of the aldose reductase inhibitor, EBPC. Cells treated for 24 hours with 0.25μg/ml doxorubicin or 30μM cisplatin in the presence of 30 or 50μM of EBPC showed a significant increase in the percentage of dead cells as compared to untreated controls (figure 3A and B). EBPC also enhanced the cytotoxic effects of doxorubicin and cisplatin on cell proliferation as evaluated by MTT assays (figure 3C and D). Treatment with EBPC alone at these concentrations had no effect on cell death or proliferation (data not shown). Furthermore, the presence of EBPC also appeared to enhance the cleavage of the PARP protein in cells treated with doxorubicin or cisplatin for 16 hours (figure 4A and B). These data suggest that inhibition of aldose reductase activity increases the effectiveness of doxorubicin and cisplatin in promoting cytotoxic effects in Hela cells.

ERK activation following treatment with doxorubicin or cisplatin is enhanced by EBPC. The ERK MAP kinase pathway has been implicated in mediating cisplatin-induced cell death [17]. To determine the effects of chemotherapeutic drugs on ERK activation, Hela cells were treated with varying concentrations of doxorubicin or cisplatin for up to 28 hours and protein lysates were immunoblotted for active ERK1 and ERK2 using a phosphorylation-specific ERK1/2 antibody. ERK proteins showed a dose dependent increase in activity in response to doxorubicin or

cisplatin (figure 5A and B). Doxorubicin and cisplatin treatment caused ERK activation within 4 hours of treatment suggesting that ERK activity is involved in early events that regulate the cellular response to these anti-cancer drugs (figure 5C and D).

We next examined whether the ERK pathway was involved in the enhanced chemotherapeutic drug-induced cell death observed in the presence of EBPC (figure 3). Cells were treated for 10 hours with cisplatin (30μM) or doxorubicin (0.25μg/ml) in the absence or presence of EBPC (30μM) only, or EBPC plus the MEK1/2 inhibitor U0126 (10μM), which prevents ERK activation. The 10 hour time point was chosen because it was after the ERK proteins were activated in response to doxorubicin or cisplatin but before the cytotoxic effects of these drugs are observed. The presence of EBPC significantly enhanced the activity of the ERK proteins in response to doxorubicin and cisplatin (figure 6A and B). EBPC-treatment alone had no effect on ERK activity (data not shown). As expected, the presence of U0126 blocked ERK activation under all conditions in figure 6. These data indicate that aldose reductase activity during exposure to doxorubicin or cisplatin suppresses ERK activation and may function to protect cell survival.

Inhibition of ERK activity reverses EBPC-induced enhancement of doxorubicin- or cisplatin-mediated cytotoxicity. Lastly, to demonstrate that ERK activity mediates the enhanced cell death observed during aldose reductase inhibition, cytotoxicity assays were performed in the presence of U0126. Cells were treated with low concentrations of doxorubicin or cisplatin in the absence or presence of 30μM EBPC and U0126 (10μM) for 2 days and the percentage of dead cells was determined following staining with trypan blue. Similar to the data presented in figure 3, treatment with EBPC significantly enhanced the cytotoxic response to both doxorubicin and cisplatin (figure 7). The presence of U0126 effectively reversed the EBPC-mediated cytotoxic

response to doxorubicin and cisplatin (figure 7). The ability of U0126 to inhibit cisplatin-mediated cell death is in support of previous studies [17]. These data support a role for ERK activation in mediating genotoxic drug-induced apoptosis in Hela cells.

Discussion

In this study we present evidence that inhibition of aldose reductase, the polyol pathway enzyme that converts glucose to sorbitol, sensitizes cancer cells to chemotherapeutic agents. These findings provide the basis for further studies of aldose reductase inhibitors as adjuvant therapy in the treatment of cancer. Importantly, aldose reductase inhibitors may prove beneficial to cancer patients by affording the use of lower doses of doxorubicin or cisplatin that would be similarly effective in promoting cancer cell death, while reducing the toxic effects of these chemotherapeutic compounds on normal cells. Other studies also provide evidence that targeting the aldose reductase enzyme may be a beneficial approach for treating cancer. For example, liver cancer cells overexpressing aldose reductase are resistant to treatment with doxorubicin and this resistance is reversible by treatment with aldose reductase inhibitors [22]. In addition, aldose reductase is expressed in cancer cell lines isolated from renal, ovarian, brain, breast, non-small cell lung, and small cell lung tissue [2] and may affect the tumor cell responsiveness to chemotherapeutic compounds. Importantly, aldose reductase inhibitor compounds are being tested in clinical trials in the United States for treating diabetic neuropathy [23] and could be rapidly transferred for adjuvant therapy in the treatment of cancer.

One potential mechanism by which aldose reductase facilitates cancer cell resistance to chemotherapeutic compounds is by affecting the transport or metabolism of anti-cancer drugs. For example, increased expression of aldose reductase may account for carbonyl reduction and detoxification of doxorubicin in a doxorubicin-resistant human stomach carcinoma cell line [24]. Thus, aldose reductase may reduce drug effectiveness by promoting the metabolism of chemotherapeutic compounds. In addition, treatment of cells with sorbitol decreased the sensitivity of non-small-cell lung cancer cell lines to cisplatin through a mechanism involving

inhibition of Na^+-K^+ ATPase activity, which appears to be important for the uptake of cisplatin [25]. These findings suggest that increased aldose reductase activity may also reduce drug uptake into cancer cells.

In addition to mediating complications associated with diabetes, aldose reductase serves a function in preserving cell integrity in stressful environments. For example, increased aldose reductase activity and concomitant increases in intracellular sorbitol concentrations are used to maintain an osmotic balance with the extracellular environment. This is best described in the kidney where renal medullary cells that are exposed to a high salt environment generate sorbitol as an organic osmolyte to maintain osmotic balance [26,27]. Aldose reductase-mediated production of sorbitol also serves as a cryopreservative and thus promotes cell survival. This is evident in overwintering insects and hibernating animals that use increased intracellular sorbitol levels to lower metabolic requirements and protect cellular functions [28,29]. Similarly, aldose reductase activity in cancer cells may act as a permissive factor in allowing other growth regulatory enzymes to function more efficiently. An alternative function for increased aldose reductase activity in cancer cells may be to provide additional substrates to meet the high-energy requirements of rapidly proliferating cells. This can be demonstrated in proliferating sperm germ cells, which use fructose derived from sorbitol as an energy source during normal metabolic processes [30].

In these studies, we examined the effects of aldose reductase inhibition on the activity of the ERK signaling pathway and its involvement in cell survival following treatment with low dose of doxorubicin or cisplatin. Both cisplatin and doxorubicin have been reported to cause activation of the MAP kinase pathways [17,31,32]. Furthermore, ERK and p38 MAP kinase activation occurs in response to increased extracellular osmolarity following sorbitol treatment

[33]. Our results demonstrate that aldose reductase inhibition enhances ERK activity following treatment with doxorubicin and cisplatin in Hela cells (figure 6A and B). Thus, ERK activation appears to mediate cell death induced by aldose reductase inhibition. Consistent with this, the addition of a MEK inhibitor to block ERK activity reversed the increase in doxorubicin or cisplatin-induced cell death observed in the presence of the aldose reductase inhibitor (figure 7). The mechanism by which aldose reductase inhibition activates ERK is not clear but may involve changes in metabolism or transport of doxorubicin and cisplatin. These possibilities are currently being investigated.

ERK activation following treatment with chemotherapeutic agents may function in inhibiting or promoting a cell death response depending on the cell type and stimulus. Our findings suggest that ERK activation promotes cell death and is in agreement with other studies examining cisplatin-induced cell death in Hela cells [17]. Further support of ERK activation as a promoter of apoptosis is demonstrated in neuronal cells, where ERK activation by taxol causes phosphorylation of the microtubule associated protein Tau and promotes apoptosis [34]. In addition, a novel vitamin K analog inhibits MCF7 growth through a mechanism that may involve enhanced ERK protein phosphorylation [35]. The mechanisms by which ERK proteins promote an apoptotic response are not fully defined. However, one possibility suggests that ERK activation in response to cisplatin stabilizes the p53 tumor suppressor protein and its function in promoting apoptosis [36].

In contrast, other reports suggest that the ERK pathway is not involved or protects against genotoxic-induced cell death. For example, a lack of involvement is suggested in some carcinoma cell lines where doxorubicin treatment does not activate the ERK pathway [31]. However, inhibition of the ERK pathway sensitizes ovarian carcinoma cells to cisplatin-mediated

cytotoxicity indicating that the ERK pathway may also serve a protective function against genotoxic stress [37, 38, 39]. Similarly, activated Raf-1, an indirect upstream activator of ERK proteins, protects against apoptosis and deletion of the activated Raf-1 sensitizes cells to apoptosis [40]. However, the mechanism by which Raf-1 inhibits apoptosis appears to be through targeting and inactivating of the anti-apoptotic Bcl-2 protein at the mitochondrion and not by activating ERK proteins [41]. Thus, ERK's role in regulating cellular responses may be distinct from Raf-1 and may function in promoting or inhibiting an apoptotic event in a cell- and stimuli-dependent manner.

In summary, our studies provide additional support for the use of aldose reductase inhibitors as adjuvant therapy for treating cancer. This may have significant implications in regards to reducing chemotherapy-related drug toxicity without a loss in the effectiveness of these anticancer agents in eliminating cancer cells. Further studies in animal models are required to determine whether aldose reductase inhibitors could be effective adjuvants in cancer chemotherapy.

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Figure legends

Figure 1. **Effect of doxorubicin or cisplatin on cytotoxicity of Hela cells.** The percentage of dead cells was determined following incubation with varying concentrations of cisplatin (**A**) or doxorubicin (**B**). Detached cells and adherent cells were collected, stained with trypan blue, and counted using a hemocytometer after 1 (closed squares) or 2 (open squares) day incubation with the drugs. The data represents averages and standard deviations from 4 separate culture dishes. Cell proliferation was also determined using the MTT assay in cells treated with varying concentrations of cisplatin (**C**) or doxorubicin (**D**) for 1 (closed squares) or 2 (open squares) days. Day 1 samples contained fewer cells, hence the lower absorbance readings in the untreated condition as compared to day 2 cells. Data represents average and standard deviations form 3 separate culture dishes.

Figure 2. **Effect of doxorubicin or cisplatin on poly (ADP-ribose) polymerase (PARP) cleavage.** Cells were incubated with the indicated concentrations of doxorubicin (Dox., panel **A**) or cisplatin (Cispl., panel **B**) for 24 hours. Time course of PARP cleavage following exposure to 1μg/ml doxorubicin (**C**) or 60μM cisplatin (**D**). Cell lysates were collected and the proteins were separated by SDS-PAGE and immunoblotted for PARP. Arrows indicate the 85 kD cleaved form of PARP that is generated following apoptotic stimuli. The data are representative of at least 3 separate experiments.

Figure 3. **Aldose reductase inhibitors enhance the cytotoxic response to doxorubicin or cisplatin.** Cells were incubated with 0.25μg/ml doxorubicin (**A**) or 30μM cisplatin (**B**) for 24 hours in the absence or presence of the aldose reductase inhibitor EBPC (30 or 50μM). Detached and adherent cells were collected and the percentage of dead cells was determined following staining with trypan blue. Data are representative of 2 separate experiments each done

in quadruplicate. Cell proliferation was determined using MTT assay following treatment for 2 days with varying doses of doxorubicin (**C**) or cisplatin (**D**) in the absence or presence of 0-60μM EBPC. The difference between non-treated and EBPC treated cells was statistically significant by analysis of variance ($p<0.05$).

Figure 4. **Inhibition of aldose reductase enhances PARP cleavage.** (**A**) PARP cleavage immunoblots in cells were left untreated or exposed to 0.25μg/ml doxorubicin (Dox, left panel) or 30μM cisplatin (Cis, right panel) in the absence or presence of EBPC (60μM) for 16 hours. (**B**) Cells were treated with varying concentrations of cisplatin in the absence or presence of 30μM EBPC. Arrows indicate the 85 kD cleaved form of PARP that is generated following apoptotic stimuli.

Figure 5. **Activation of ERK1 and ERK2 in cells treated with doxorubicin or cisplatin.** Cells were incubated with varying doses of doxorubicin (**A**) or cisplatin (**B**) for 20 hours and protein lysates were collected and immunoblotted for active ERK1 and ERK2 (ppERK1/2, top panels) and total ERK2 (bottom panels) for a protein loading control. The time course for ERK activation (ppERK1/2, top panels) in response to 0.5μg/ml doxorubicin (**C**) or 60μM cisplatin (**D**). Total ERK2 is shown for a protein loading control in the bottom panels.

Figure 6. **Inhibition of aldose reductase enhances ERK activity.** Cells were incubated with 0.25μg/ml doxorubicin (**A**) or 30μM cisplatin (**B**) and EBPC (30μM) for 10 hours in the absence or presence of the MKK inhibitor, U0126 (10μM). Cell lysates were collected and immunoblotted for active ERK proteins (ppERK1/2, top panels) and total ERK2 for a protein loading control (bottom panels). *Lane 1* indicates doxorubicin or cisplatin only treated cells. *Lane 2* indicates doxorubicin or cisplatin plus EBPC-treated cells. *Lane 3* indicates doxorubicin or cisplatin plus EBPC and U0126-treated cells.

Figure 7. **ERK activation mediates EBPC-induced cell death in response to low doses of doxorubicin or cisplatin.** The percentage of dead cells was determined following a 2 day incubation with 0.1μg/ml doxorubicin or 30μM cisplatin in the absence or presence of EBPC (30μM) and U0126 (10μM). Both floating cells and adherent cells were collected and the percentage of dead cells was counted following staining with trypan blue. The percentage of dead cells treated only in the presence of EBPC or U0126 was similar to the untreated controls. Values represent the averages and standard errors from 3 separate experiments. The difference between non-treated and EBPC treated cells was statistically significant as determined by analysis of variance ($p<0.05$).

Figure 1.

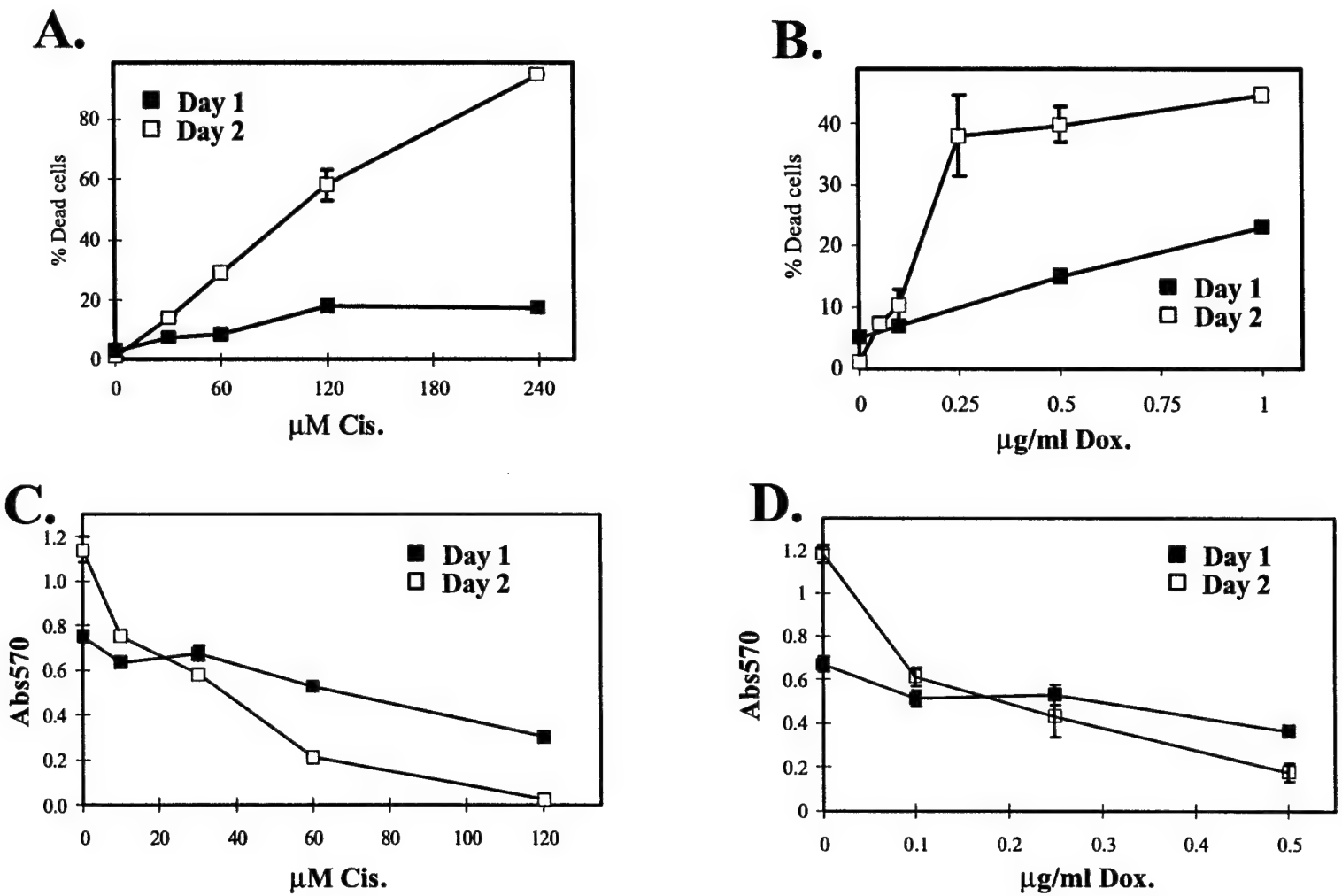

A.
Day 1
Day 2
% Dead cells
0
20
40
60
80
0
60
120
180
240
µM Cis.
B.
Day 1
Day 2
% Dead cells
0
10
20
30
40
0
0.25
0.5
0.75
1
µg/ml Dox.
C.
Day 1
Day 2
Abs570
0.0
0.2
0.4
0.6
0.8
1.0
1.2
0
20
40
60
80
100
120
µM Cis.
D.
Day 1
Day 2
Abs570
0
0.2
0.4
0.6
0.8
1
1.2
0
0.1
0.2
0.3
0.4
0.5
µg/ml Dox.

Figure 2.

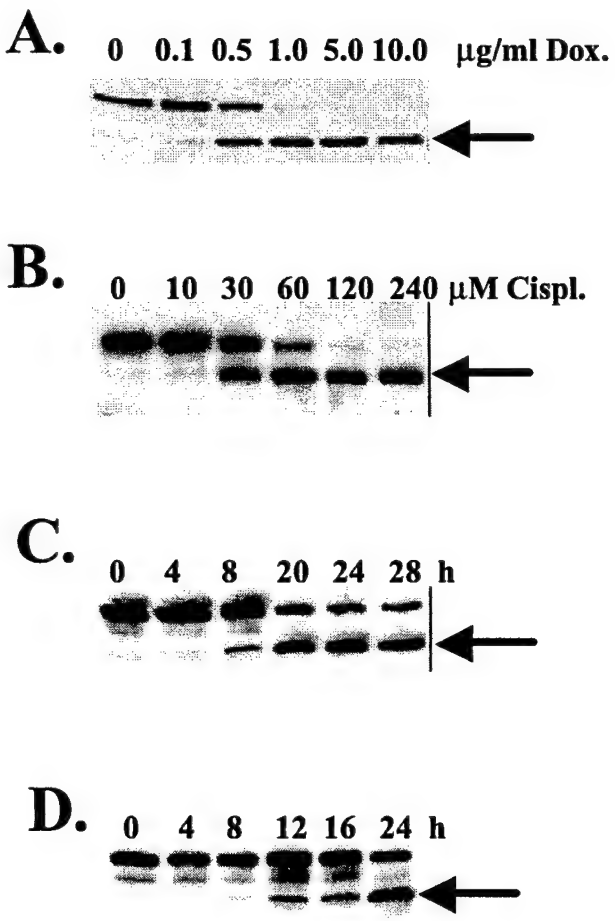

A.
0 0.1 0.5 1.0 5.0 10.0 μg/ml Dox.
B.
0 10 30 60 120 240 μM Cispl.
C.
0 4 8 20 24 28 h
D.
0 4 8 12 16 24 h

Figure 3.

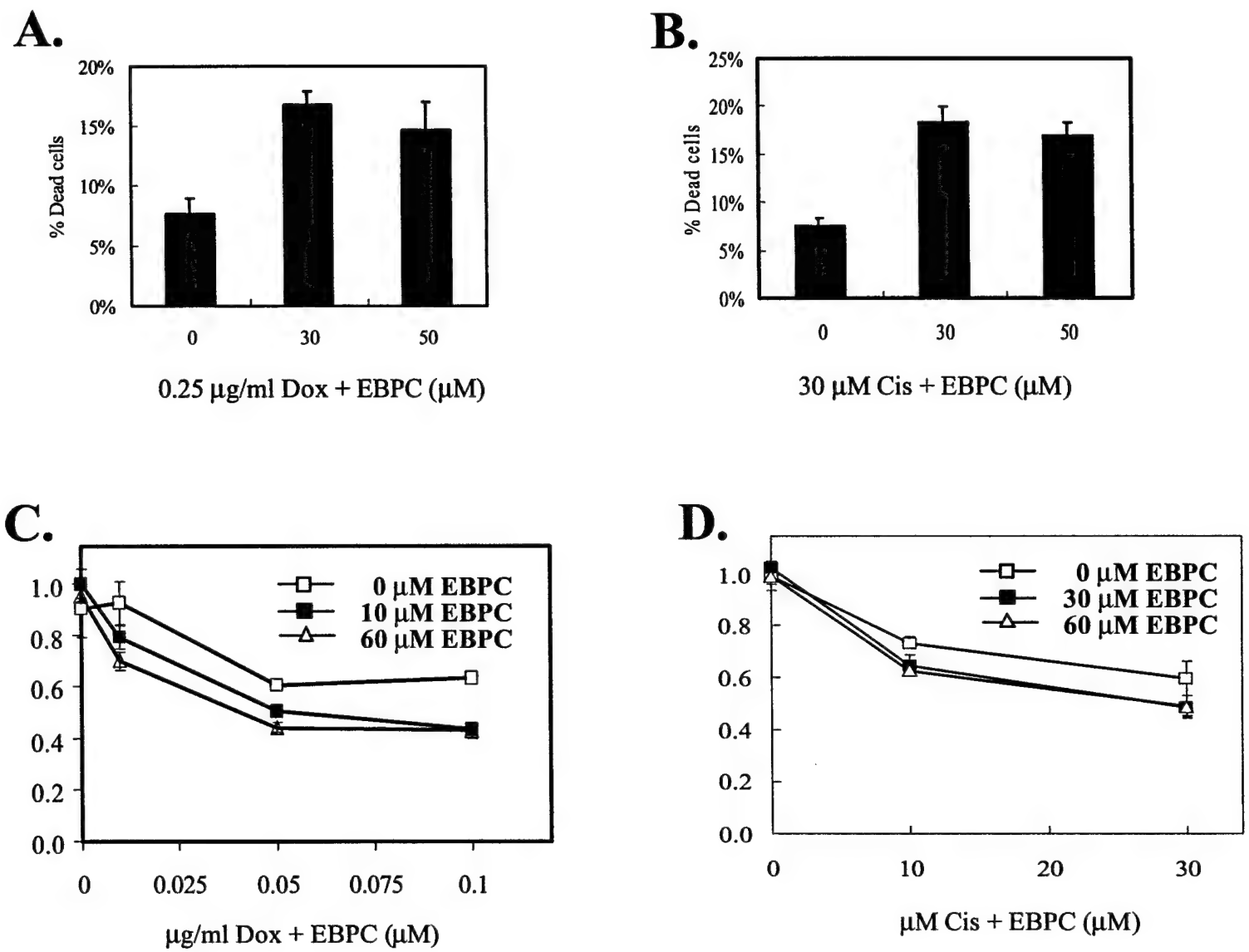

A.
20%
15%
10%
5%
0%
% Dead cells
0
30
50
0.25 µg/ml Dox + EBPC (µM)
B.
25%
20%
15%
10%
5%
0%
% Dead cells
0
30
50
30 µM Cis + EBPC (µM)
C.
1.0
0.8
0.6
0.4
0.2
0.0
0 µM EBPC
10 µM EBPC
60 µM EBPC
0
0.025
0.05
0.075
0.1
µg/ml Dox + EBPC (µM)
D.
1.0
0.8
0.6
0.4
0.2
0.0
0 µM EBPC
30 µM EBPC
60 µM EBPC
0
10
20
30
µM Cis + EBPC (µM)

Figure 4.

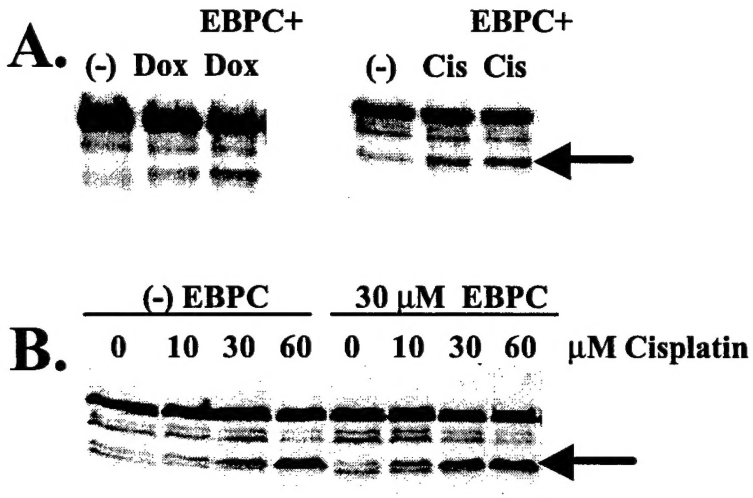

A.
(-) Dox
EBPC+ Dox
(-) Cis
EBPC+ Cis
B.
(-) EBPC
30 μM EBPC
0 10 30 60 0 10 30 60 μM Cisplatin

Figure 5.

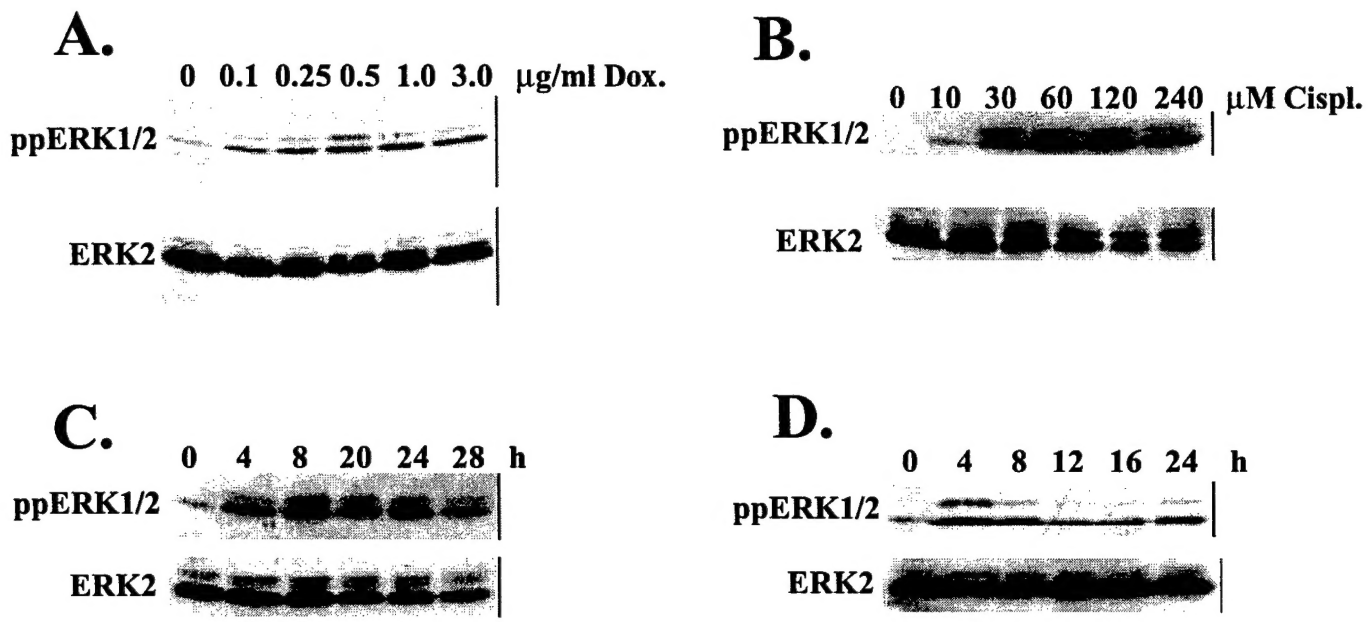

A.
0 0.1 0.25 0.5 1.0 3.0 μg/ml Dox.
ppERK1/2
ERK2
B.
0 10 30 60 120 240 μM Cispl.
ppERK1/2
ERK2
C.
0 4 8 20 24 28 h
ppERK1/2
ERK2
D.
0 4 8 12 16 24 h
ppERK1/2
ERK2

Figure 6.

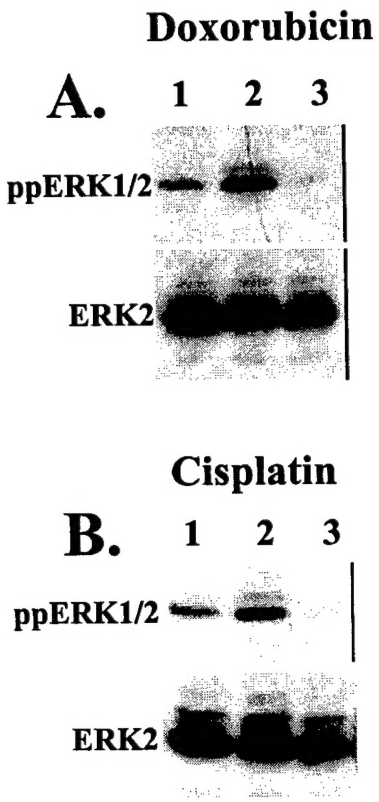
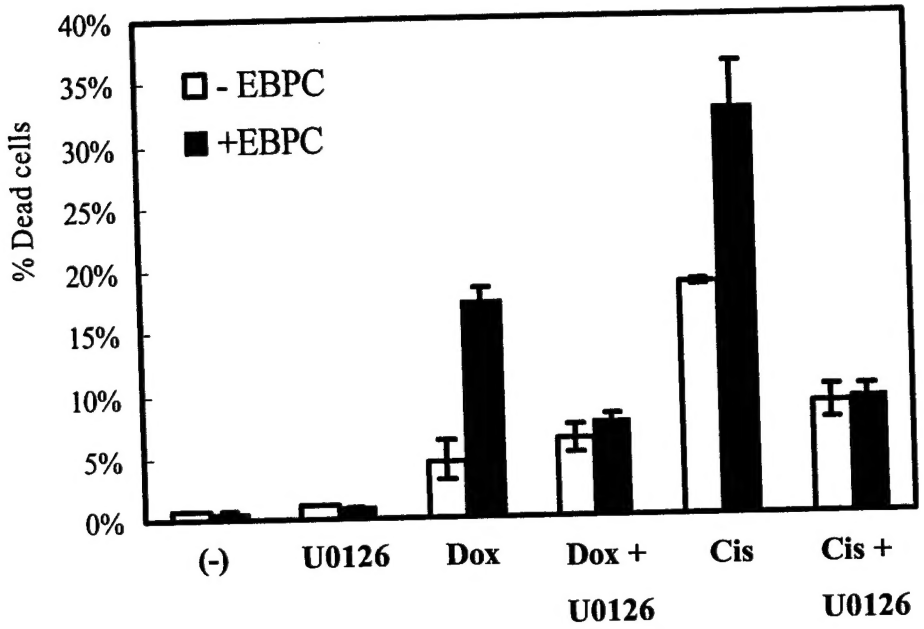

Doxorubicin
A.
1 2 3
ppERK1/2
ERK2
Cisplatin
B.
1 2 3
ppERK1/2
ERK2

Figure 7.


40%
35%
30%
25%
20%
15%
10%
5%
0%
% Dead cells
- EBPC
+EBPC
(-)
U0126
Dox
Dox +
U0126
Cis
Cis +
U0126